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14. ABSTRACT We have transformed a plant, Arabidopsis thaliana, with the bacterial genes for the three subunit proteins of the soluble methane monooxygenase and with two associated genes, the mmoG and the mmoC. The presence of the genes and the transcripts in selected lines of the transformed plants was confirmed at the genomic, transcript and protein levels. The transformed plants removed more methane compared to the wild-type, but only slightly, about 5% in 7 days. The production of 1-naphthol from naphthalene was 2-3 times greater in transformed plant lines					
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Phytoremediation of Atmospheric Methane

ABSTRACT

We have transformed a plant, *Arabidopsis thaliana*, with the bacterial genes for the three subunit proteins of the soluble methane monooxygenase and with two associated genes, the *mmoG* and the *mmoC*. The presence of the genes and the transcripts in selected lines of the transformed plants was confirmed at the genomic, transcript and protein levels. The transformed plants removed more methane compared to the wild-type, but only slightly, about 5% in 7 days. The production of 1-naphthol from naphthalene was 2-3 times greater in transformed plant lines compared to untransformed wild types. These results suggest that the sMMO was properly assembled and partially functional, however, additional controls are needed to confirm. We discuss the results and suggest follow-on experiments.

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Phytoremediation of Atmospheric Methane

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April 15, 2013

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Summary

Methane monooxygenases (MMOs) catalyze the oxidation of methane to methanol and enable methanotrophic bacteria to utilize methane as a carbon and energy source. One soluble MMO (sMMO) system is expressed by some methanotrophs under conditions of low copper concentration and consists of three components, MMOR (a reductase), MMOB (a regulatory protein) and MMOH, which contains the catalytic center. The hydroxylase MMOH is a 251 kDa dimer of three subunits (α , β and γ) in an $\alpha_2\beta_2\gamma_2$ configuration. The α subunit, encoded by the gene *mmoX* in *Methylosinus trichosporium*, contains a dinuclear iron center, where methane oxidation takes place. The β subunit (coded by gene *mmoY*) and γ subunit (coded by gene *mmoZ*) are required for the formation of the active MMOH. The genes for MMOH are clustered in an operon, which also includes *mmoB*, *mmoD* and *mmoC*, which code for MMOB (putative electron transfer protein), a protein with unknown function and MMOR (reductase), respectively (Merkx, Kopp et al. 2001). *MmoG*, a gene encoding a GroEL homologue, lies 5' of the structural genes for the sMMO enzyme in *Methylosinus trichosporium* OB3b and is necessary for smmo gene transcription and activity, perhaps as a chaperone.

Here we report that we have transformed a plant, *Arabidopsis thaliana*, with the bacterial genes for the three subunit proteins of the soluble methane monooxygenase and with two associated genes, the *mmoG* and the *mmoC*. The presence of the genes and the transcripts in selected lines of the transformed plants was confirmed at the genomic, transcript and protein levels. The transformed plants removed more methane compared to the wild-type, but only slightly, about 5% in 7 days. The production of 1-naphthol from naphthalene was 2-3 times greater in transformed plant lines compared to untransformed wild types. These results suggest that the sMMO was properly assembled and partially functional, however, additional controls are needed to confirm. We discuss the results and suggest follow-on experiments.

Introduction

The three primary greenhouse gases produced by human activity are carbon dioxide, methane and nitrous oxide. Much attention has been directed toward CO₂ mitigation; however over a time frame of 100 years CH₄ has 23 times the per molecule warming potential of CO₂, so, on a molecule-by-molecule basis, removal from the atmosphere of methane would have a greater impact than removal of an equivalent amount of CO₂ on global warming. If atmospheric CH₄ (natural and anthropogenic) were reduced to near zero, the increase in radiative forcing of climate warming caused by human activity could be reduced by up to 33%. Therefore, an effective and inexpensive method to remove CH₄ from the atmosphere could have significant effects on warming caused by greenhouse gases.

Sinks for CH₄ consist primarily of photodegradation in the upper atmosphere and biological degradation. Chemical/physical methods for degrading these relatively stable gases may be possible, but are unlikely to be economic or practical in an engineering sense. Biological methods of catalyzing CH₄ degradation have greater potential at the large scales necessary for a global effect. Methane can be oxidized by

anaerobic and aerobic microbial communities. Anaerobic oxidation of CH₄ takes place in environments that have restricted access to atmospheric gases such as oxygen and trace atmospheric CH₄. Aerobic biodegradation of CH₄ is due to a few groups of bacteria that possess a methane monooxygenase (MMO), the methanotrophs, or the closely related ammonia monooxygenase, expressed by the nitrifiers. .

Biological degradation offers the greatest potential for increasing the removal of CH₄ from the atmosphere, but increasing microbial activities is problematic because atmospheric levels of CH₄ are too low to support significant populations of methanotrophs. Since total enzymatic activity is the product of expression and biomass, a method of increasing either activity or biomass could be used to increase biodegradation of CH₄. We propose that a technology to express MMO constitutively in plants that can be grown to high biomass under cultivation could be used to remove CH₄ from the atmosphere at rates large enough to stop its increase in the atmosphere and to force down its concentrations, reducing radiative forcing and greenhouse warming by at least 18%.

To remove CH₄ from the atmosphere we propose to express bacterial methane monooxygenase (MMO) in the leaves of plants. There are two types of MMO, soluble (cytosolic, sMMO) and particulate (membrane bound, pMMO). The soluble MMO has been expressed in non-methanotrophic bacteria, albeit at low levels of activity (Jahng and Wood 1994; Jahng, Kim et al. 1996; Wood 2002). Recently pMMO has been expressed in recombinant non-methanotrophic bacteria (Gou, Xing et al. 2006). Plants expressing particulate MMO would oxidize atmospheric CH₄ to trace levels of methanol followed by degradation of the methanol *in planta* or by epiphytic bacteria that oxidize existing methanol emissions.

Plants clearly influence atmospheric CO₂ levels by taking up carbon from the atmosphere and photosynthetically fixing it into their tissues. To calculate the atmospheric conductance or mass transfer coefficient in vegetated fields of maize we used published data for CO₂ flux. CH₄ has a convective diffusivity about 11% greater than CO₂ (Table 1). The average net atmospheric and stomatal mass transfer coefficients for CO₂ are also shown in Table 1.

Table 1. Molecular and Convective Diffusivities and Net Atmospheric and Stomatal Mass Transfer Coefficients of Methane.

Compared to average empirical values for Net Atmospheric and Stomatal Mass Transfer Coefficients of photosynthetic CO₂ uptake through aerodynamic and leaf boundary layers and the stomata of maize at field scale as determined by continuous stable isotope measurements to partition net ecosystem CO₂ exchange, 0.21 m/s or 7.6 x 10⁶ μmol CO₂ / ha /h / ppmv and the coefficient of molecular diffusivity of CO₂ in air, 0.165 cm²/s (Massman 1998).

	CH ₄	Units
Coefficients of molecular diffusivity in air	0.192	cm ² /s
Ratio of molecular diffusivity to that of CO ₂ , R _{CO2}	1.16	
Ratio of convective diffusivity, R _{CO2} ^{0.667} (Campbell and Norman 1998)	1.11	
K _{mt} , mass transfer coefficient,	8.36 x 10 ⁶	μmol / ha /h / ppmv

To calculate the inter-stomatal concentrations of CH₄ and its resultant fluxes, we set the net atmospheric and stomatal mass transport flux expression equal to the areal enzymatic activity given by the observed kinetic parameters for MMO in bacteria and the plant aboveground biomass.

$$K_{mt} \left(C_b - \frac{C_{es,aq}}{K_H} \right) = \frac{V_{max} X_{area} C_{es,aq}}{K_S + C_{es,aq}}$$

Where K_{mt} = net atmospheric and stomatal mass transfer coefficient

C_b = bulk atmospheric concentration

$C_{es,aq}$ = aqueous concentration at the enzyme surface

K_H = Henry's Law constant

V_{max} = maximum substrate uptake rate

X_{area} = Areal biomass density, shoot biomass per area, average for growing season

K_S = Half saturation coefficient.

Since $C_{es,aq} \ll K_S$, this expression simplifies to a first-order relationship:

$$K_{mt} \left(C_b - \frac{C_{es,aq}}{K_H} \right) \cong \frac{V_{max} X_{area} C_{es,aq}}{K_S}$$

From which we can solve for $C_{es,aq}$ and the flux:

$$C_{es,aq} = \frac{K_{mt} C_b}{\frac{V_{max} X_{area}}{K_S} + \frac{K_{mt}}{K_H}}$$

$$Flux = K_{mt} C_b \left(1 - \frac{1}{\frac{V_{max} X_{area} K_H}{K_S K_{mt}} + 1} \right)$$

Table 2 shows V_{max} and K_S parameters reported for MMO and the seasonal and biomass assumptions for uptake of CH₄ by genetically modified field corn.

Table 2. Biological and Physical Parameters and Calculated Flux of CH₄ into GM Corn Fields in US

Assumptions: Biomass density, seasonal average shoot biomass, $X_{\text{area}} = 9.27 \times 10^6$ g biomass DW/ha (one half of the final crop shoot biomass), plants are metabolically active only 18 h/d, season length = 100 d/yr, and the total area in US planted in GM corn = 27.5×10^6 ha

	CH ₄	Units
Specific enzymatic activity for pMMO, V _{max}	1922 ^a	μmol/g biomass DW/h
Half-saturation coefficient, K _s , for pMMO	8.3 ^a	μM
Henry's Law constant, K _h	0.0013	M/atm
Atmospheric concentration, C _b	1.7	ppmv
Calculated aqueous concentration at enzyme surface, CES _a	0.00166	μM
Calculated gaseous concentration at enzyme surface, CES	1.27	ppmv
Calculated flux	3.56×10^6	μmol/ha/h
Calculated annual flux	102	kg/ha/yr
Calculated annual uptake if expression in all US GM corn	2.8	Tg/yr

^a(Lontoh and Semrau 1998), ^b (Kristjansson and Hollocher 1980)

If strong MMO expression *in planta* were accomplished, annual fluxes to genetically modified (GM) crops could be on the order of 102 kg CH₄ per hectare. If 28 million ha of feed and ethanol corn (US farmland planted in GM corn in 2007) expressed MMO, then the annual uptake by this crop alone would be about 2.8 Tg CH₄. Compare this potential removal to the annual increase in CH₄ of perhaps 6 Tg (Houweling, Kaminski et al. 1999). Additional areas planted for atmospheric phytoremediation would drive down the concentration of CH₄. Minimizing the concentrations of CH₄ in the atmosphere would reduce total radiative forcing by up to 18%, cutting the total increase in radiative forcing caused by anthropogenic emissions by one third, reducing global warming.

Importantly this technology would be inexpensive, probably much cheaper than any other greenhouse gas reduction technology. GM seed sells for an \$800 million premium in the present GM corn market. A mandate for the addition of MMO and genes to GM corn in the US could be supplemented with a subsidy for participating farmers, at say 10% of the GM premium, about \$80 million per year. So CH₄ could be removed for \$28/t CH₄ removed or about \$1.24/t CO₂ equivalent. Compare to the present value of sequestration on carbon markets: \$4-10/t CO₂.

Balance of Risk versus Benefits

Successful expression and assembly of the complex metalloenzymes, MMO, in plants will be challenging. MMO has only recently been heterologously expressed in non-methanotrophic bacteria and its activity is uncertain. However, metalloenzymes such as mammalian P450 2E1 monooxygenase and a bacterial P450 (Rylott, Jackson et al. 2006) with a fused flavodoxin redox partner have been successfully expressed in plants with high activity. High expression of MMO in plants will require intensive work with some risk, but the potential benefits in terms of reduced greenhouse gas levels and greatly reduced radiative forcing are enormous.

Reduction of atmospheric CH₄ will reduce greenhouse warming, giving human-kind additional time to switch to a non-carbon based energy system, and providing a counter to the possibility of rapid methane release. But it is important to realize that even if methane levels and global warming are decreased, other detrimental effects directly attributable to CO₂ will continue. Most important among these is acidification of the surface ocean. Acidification and ultimately warming due to CO₂ alone must be addressed by removing CO₂ from the atmosphere and prevention of further emissions of CO₂.

Because the trace levels of CH₄ are so low and support minimal ecosystems, we expect the ecological effects of decreasing CH₄ levels to be minimal. Atmospheric trace CH₄ may support some surface communities of methanotrophic bacteria and those communities may be negatively impacted by decreasing concentrations of CH₄ in the bulk atmosphere. Thus reduction of CH₄ to preindustrial levels can be viewed as environmentally benign.

We are proposing a radical technology with very large potential payoffs and unpredictable risks. But it is important to remember that we are not proposing an immediate global implementation of transgenic phytoremediation; rather we are proposing a novel idea at the proof-of-concept stage. If we can show that MMO can be functionally expressed in a lab plant such as *Arabidopsis*, then a consideration of the risk and benefits can be undertaken by the scientific community and ultimately by the public, with a clearer understanding of the characteristics and capabilities of the transformed plants in hand.

Objectives

Our objective was to demonstrate the functional expression of bacterial genes for the soluble methane monooxygenase in plants.

Specific objectives were:

1. Development of vectors for the essential subunits of soluble MMO genes, incorporating strong constitutive promoters for nuclear chromosomal plant expression
2. Development of vectors for mmo genes for operon expression in chloroplast chromosome
3. Transformation of *Arabidopsis thaliana* with the bacterial mmo genes, in both nuclear and chloroplast genomes.
4. Determination of expression at the DNA, mRNA, and protein levels, and determining CH₄ uptake rates by the plants.

Accomplishments

Multiple expression vectors for nuclear transformation of *Arabidopsis* with smo genes.

The aim of this project was to express active sMMO in plants. Towards this goal, mmoX, mmoY, mmoZ, mmoB, mmoD, mmoC, and mmoG genes were synthesized with codon usage optimized for *Arabidopsis thaliana* (*Arabidopsis*), and the GC content set to an average of 45 %. In addition, a translation enhancer (AACA) was introduced adjacent to the ATG. The genes were synthesized with the coding for the FLAG-Tag (ATG GAC TAC AAG GAT GAC GAT GAC AAG) inserted before the ATG start codon of each gene. The peptide sequence of the FLAG-tag is: N-DYKDDDDK-C (1012 Da). A FLAG-tag (Einhauer and Jungbauer

2001) can be used in many different assays that require recognition by an antibody. If there is no antibody against the studied protein, adding a FLAG-tag to the protein allows the protein to be visualized with an antibody against the FLAG sequence.

The optimized sequences are shown in the Appendix.

Co-transformation with separate vectors

In a different approach we attempted to transform *Arabidopsis* using separate binary vectors, each of which contained a separate mmo gene. *Arabidopsis* was infected the three vectors simultaneously based on the observation that one infection event made it more likely that a second and perhaps third infection would occur.

The genes for the α , β and γ mmo subunits (mmoX, mmoY, and mmoZ, respectively) were transformed separately into the intermediate, cloning vector pART7 and then into three binary vectors (α into pART27, β into pMLBart, and γ into pJ0530). In two attempts, *Arabidopsis* plants were transformed with these vectors simultaneously. We obtained twenty-four primary transformants for pART27- α , but only 1 putative transformant for pMLBart- β , and no transformants for pJ0530- γ . We re-transformed the *Agrobacterium* strain GV3101 separately with these constructs, and confirmed their presence by PCR (not shown). Thus, the co-transformation approach was abandoned.

Transient expression

We also attempted transient expression of MMO subunits in *Nicotiana benthamiana* through agro-infiltration. The mmoX, mmoY and mmoZ genes were cloned into the pEAQ-HT-DEST2 vector by using the Gateway recombination (Sainsbury, Thuenemann et al. 2009). This vector possesses an N-terminal his-tag to facilitate purification and detection of the resulting his-tagged proteins.

The three plasmids were transformed into cells of *Agrobacterium* GV3101, which were introduced into the young leaves of 6-week old *Nicotiana benthamiana* plants. RT-PCR indicated that all three genes were transcribed; however, no protein was visible in leaf extracts when analyzed by SDS-PAGE and westerns using anti-his antibodies. Subsequent DNA analysis revealed that the pEAQ-HT-DEST 2 vector carries a single nucleotide insertion which changes the reading frame downstream of the cloned gene, but after the his-tag, therefore protein cannot be detected using anti-his antibodies. To resolve this problem it was decided to separately add a his-tag to the mmoX, mmoY and mmoZ genes and then re-clone these genes into the plant expression vector pEAQ-HT-DEST1. Agro-infiltration of *Nicotiana benthamiana* was performed with these new constructs; however, no protein was detected on SDS-PAGE gels by western analysis using anti-his antibodies. Thus, attempts at transient expression were abandoned.

Antibodies to the sMMO subunits

Antibodies specific to the MMO subunits are needed in order to visualize the proteins electrophoretically and to study their expression and assembly in plants. To generate protein to raise antibodies to the MMO subunits, the mmoX, mmoY and mmoZ genes were subcloned into the pET-YSB LIC 3C (LIC) vector and expressed as the his-tagged constructs in *Escherichia coli* Rosetta 2 cells. Although the expression was strong, the protein produced inclusion bodies and was localized in the

insoluble fraction. Cell lysis was repeated but using a phosphate buffer containing 7 M urea to solubilize the proteins, which were then purified by using nickel-affinity resin (pre-equilibrated in the urea buffer). As shown in Figure 1, in two attempts, sufficient purified protein was obtained for antibody production from mmo α and mmo γ , but not mmo β .

Design and development of stacked vectors for expression of sMMO subunits, MMOx, MMOY, and MMOZ

Since simultaneous transformation with separate vectors containing mmoX, mmoY and mmoZ genes was unsuccessful, as was transient expression; we focused on the construction of stacked vectors containing the three subunit genes for the sMMO and kanamycin resistance as a selection marker. We also constructed a separate multi-gene vector containing mmoC and mmoG under control of a second selection marker, resistance to hygromycin. Lines positive for transformation by the first stacked vector were transformed a second time with the second stacked vector, producing lines transformed with all five mmo genes: mmoX, mmoY and mmoZ, mmoC and mmoG.

The complexity of the multiple stacked vector design for nuclear transformation (objective 1) led us to abandon objective 2, development of vectors for mmo genes for operon expression in chloroplast chromosome of Arabidopsis.

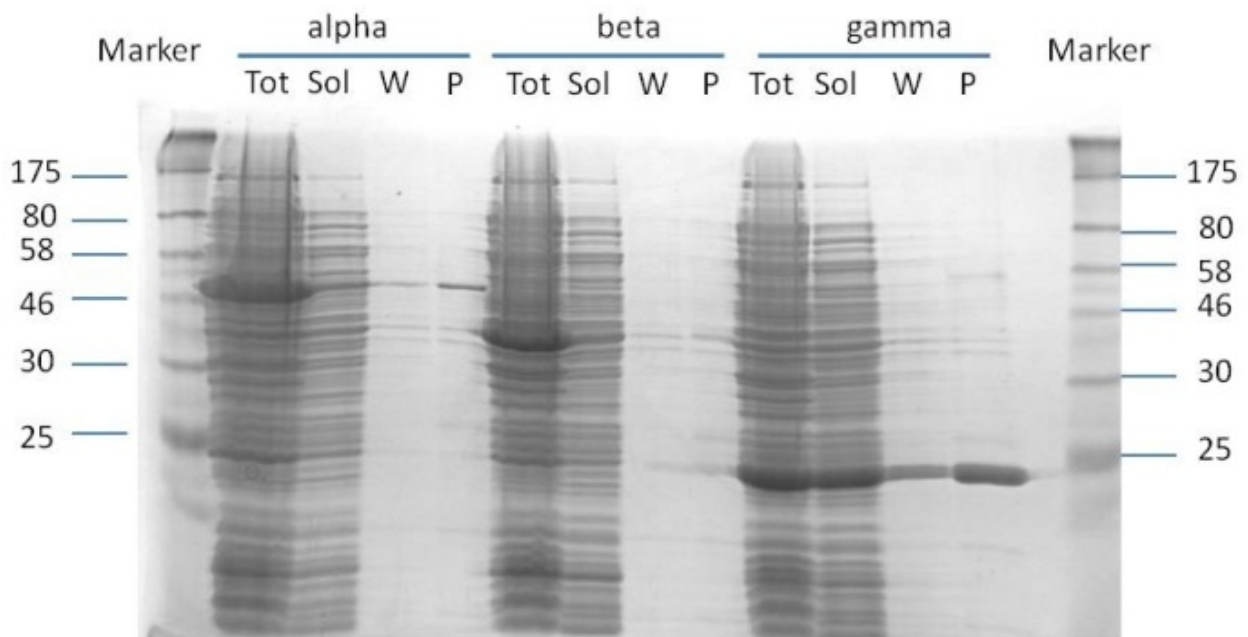


Figure 1. SDS-PAGE analysis of the purification of the MMO subunits. Sufficient purified protein (lanes P) for antibody production was collected from elute protein of mmo α and mmo γ , but not mmo β .

Key: Tot=total protein; Sol=supernatant (soluble in urea buffer); W=wash step with pH 6.3 (instead of low imidazole); P=elute protein with pH 4.5 (instead of high imidazole)

To construct the stacked vectors we used the pSAT system of plant transformation vectors with multiple restriction sites into which can be inserted up to five foreign genes (Figure 2) (Tzfira, Tian et al. 2005). Our vector construction strategy was to construct cloning vectors in five versions, pLZ1 through pLZ5 (Table 3), each with one of four distinct promoters, all of which are proven strong promoters in Arabidopsis. Separate, distinct promoters were used in order to minimize the likelihood of gene silencing.

Table 3 – Elements of the cloning vectors used in the construction of the stacked transformation vectors

Vector (plzn)	promoter	5'utr	3'utr	8 base site
pLZ1	psba	psba	psba	AsiSI
pLZ2	Rrn	rbcl	rbcl	Ascl
pLZ3	rbcl	rbcl	rbcl	FseI
pLZ4	rrn	atpb	Rps16	NotI
pLZ5	rrn	T7gene 10	petD	PmeI

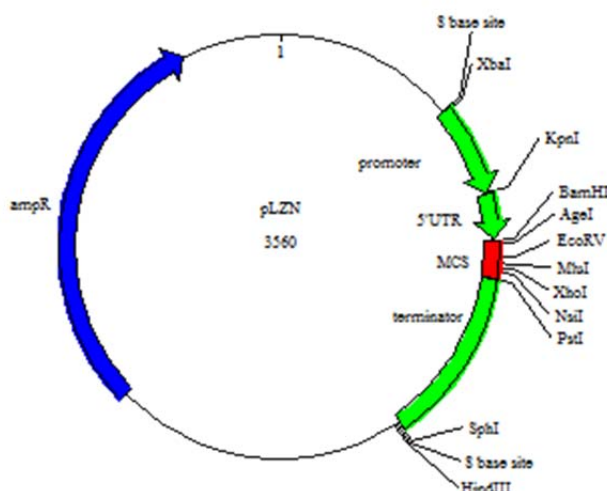


Figure 2 – Schematic of the pSAT cloning vector

Multiple expression vectors for nuclear transformation of Arabidopsis with mmo genes.

The mmo genes were flanked with EcoRI site at 5' end and BamHI site at 3' end for construction into a modular series of clone vectors, pSATs. Then the expression cassette of these genes were cut from the pSATs and integrated into binary vector pPZP-RCS2-nptII and pPZP-RCS2-hptII to produce Prcs2-nptII-3X-4Y-6Z (Figure 3) and prcs2-hptII-4C-6G (Figure 10). Correct insertion of the genes into pRCS2-nptII-XYZ was shown by differential digestion with specific homing endonucleases (Figure 4).

Completion of the triple vector construction of mmoX, Y and Z in *E. coli* was confirmed by PCR and sequencing. After confirmation the vector was transformed into *Agrobacterium*, as confirmed by PCR.

Arabidopsis was transformed by the floral dipping technique (Clough and Bent 1998). T₁ seeds from these plants were allowed to germinate on selection medium, containing 1/2MS plus 50 mg/l kanamycin (Figure 5).

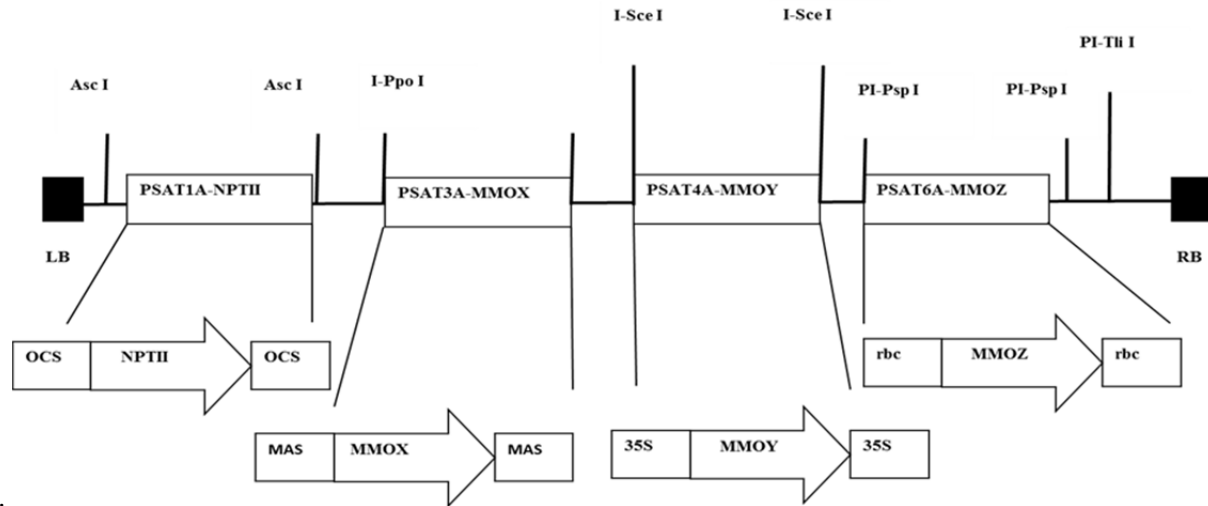


Figure 3. Construction of pRCS2-NptII-3X-4Y-6Z containing mmoX, mmoY and mmoZ and the selection marker gene nptII, for kanamycin resistance.

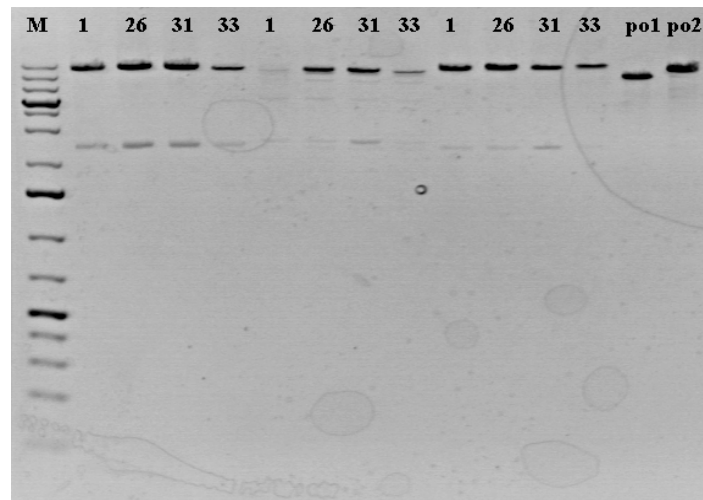


Figure 4. Digestion of pRCS2-nptII-XYZ with Homing Endonucleases to confirm the construction. Lanes 1, 26, 31, 33 represent the clones of *E. coli* Dh5a containing the vector pRCS2-XYZ. M: 1kb DNA ladder; lane 2-5: the vectors were digested with I-SceI; lane 6-9: the vectors were digested with PI-PspI; lane 10-13: the vector were digested with I-PpoI; po1: the vector pRCS2-nptII was digested with I-SceI; po2: the vector pRCS2-nptII-mmoY digested with PI-pspI.

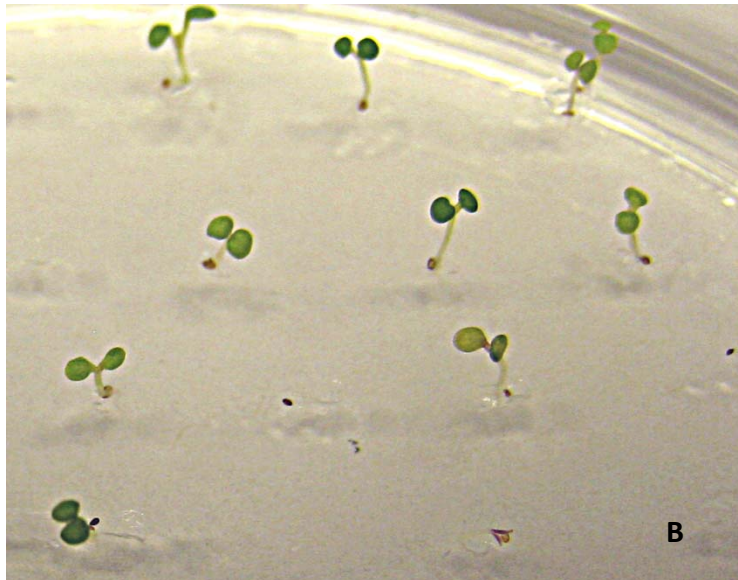


Figure 5 – Transformed Arabidopsis seedlings growing on selection medium containing 50 mg/l kanamycin

PCR to confirm the transformation of *Arabidopsis thaliana* with *mmoX*, *mmoY*, and *mmoZ*.

The PCR primed by the primers of sat6aseqf1 and sat6aseqr1 binding to the rbc promoter and terminator separately. The transformation of Arabidopsis with the gene for mmoZ was confirmed by PCR amplification of a sequence containing the full sequence of mmoZ and parts of the promoter and terminator (Figure 6)

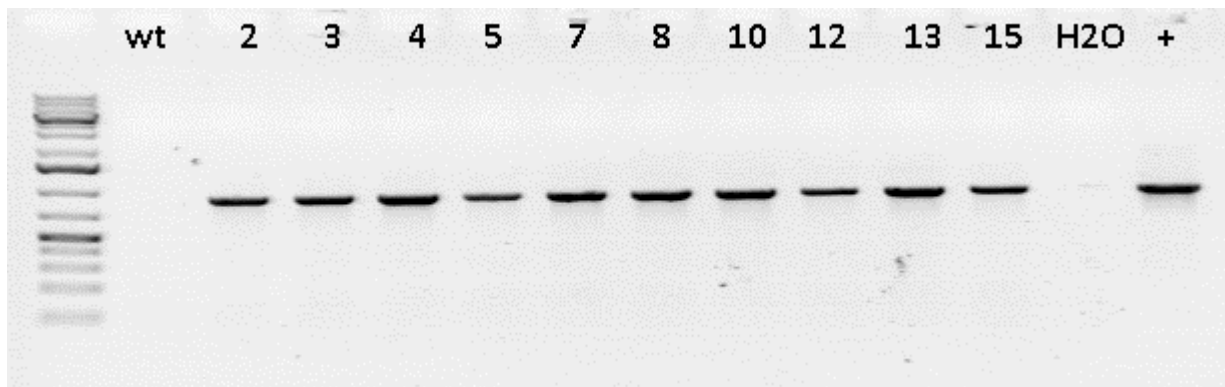


Figure 6 – Confirmation of the transformation of 10 lines of Arabidopsis transformed with the gene for mmoZ. PCR detection of the amplicon amplified with sat6aseqf1 and sat6aseqr1 binding to the rbc promoter and terminator separately. The PCR product included the full sequence of mmoZ and partial sequence of promoter and terminator. Wt, wild type Arabidopsis thaliana; 2-15, the lines with kanamycin resistance; +, positive control, pRCS2-nptII-XYZ vector

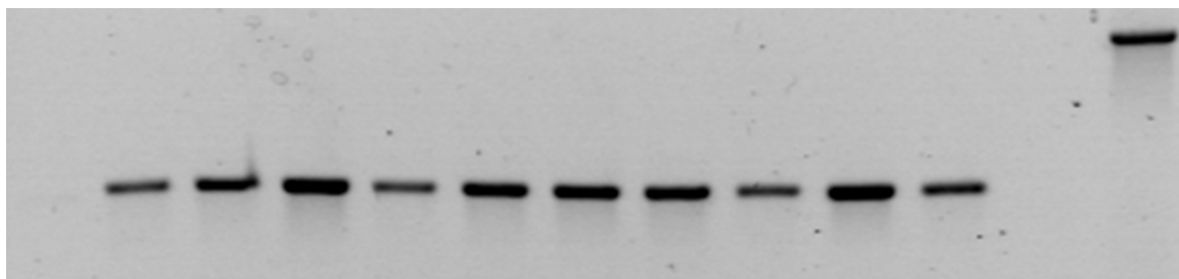


Figure 7 -- pSAT6a-mmoz amplification primed with sat6aseqF1 and sat6aseqR1

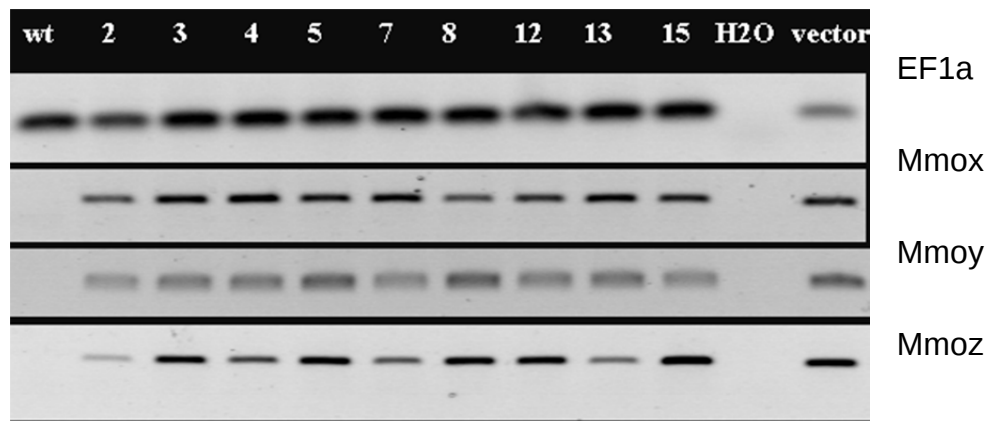


Figure 8 -- RT-PCR to confirm the expression of mmo genes in Arabidopsis. Lane 1 shows the transcript of the housekeeping gene, EF1a, but none of the transcripts of the mmoX – Z genes in the untransformed wild types, while lanes 2-10 show the expression of EF1a and the transgenes in 9 transformed lines. Lanes 11 and 12 show the RT-PCR results for a water control and the vector in E.coli.

Design and development of stacked vectors for expression of MMO auxiliary genes, C and G

Since the methane monooxygenase assembly in bacteria seems to require helper proteins beyond the three subunits of the MMO itself, it is necessary to transform plants with more than three genes of interest. We accomplished this by performing a subsequent transformation of Arabidopsis previously transformed with mmoX, Y, Z, using a second vector (Figure 9) containing the gene for the reductase protein, mmoC and the helper gene, mmoG, and the bar gene for resistance to a second selection marker, phosphinothricin (ppt). As a backup we also constructed a second vector utilizing a gene for hygromycin resistance (hptII) as shown in Figure 10. Since the bar gene coding resistance to ppt worked well, the second vector was not used.

We had intended to include mmoB, the gene for the regulator protein, Protein B, but gene synthesis problems prevented its inclusion in the second vector.

Selected lines of *Arabidopsis thaliana* expressing mmoX, Y, Z (as determined by RT-PCR) were infected by floral dipping with *Agrobacterium* harboring prcs2-bar-mmoC-mmoG and the seeds were germinated on ½ MS medium with 50 mg/L kanamycin and 5 mg/L phosphinothricin (PPT).

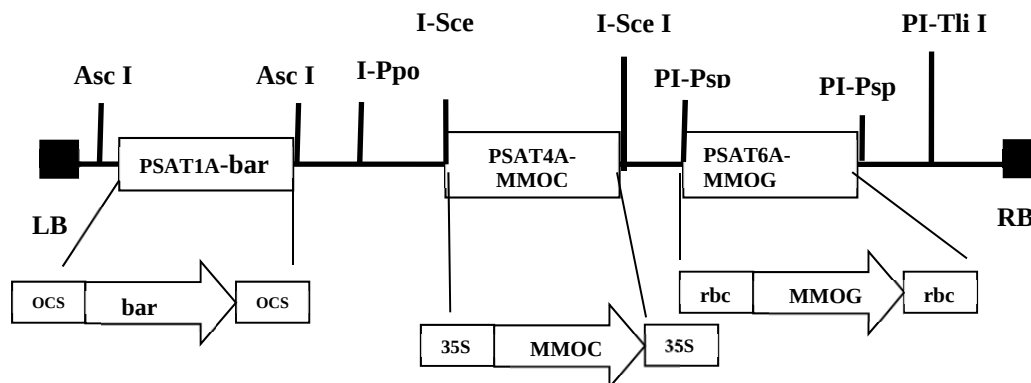


Figure 9 -- Construction of pRCS2-bar-4C-6G transformation vector. The selection marker for resistance to the herbicide phosphinothricin (ppt) was the bar gene, with the ocs plant promoter and terminator. The mmoC was under the control of the 35S promoter and terminator and the mmoG gene was under the rbc promoter and terminator. All the promoters were proven strong constitutive promoters for Arabidopsis. Also identified are the PSAT cloning vectors and the restriction sites used to construct the transformation vector.

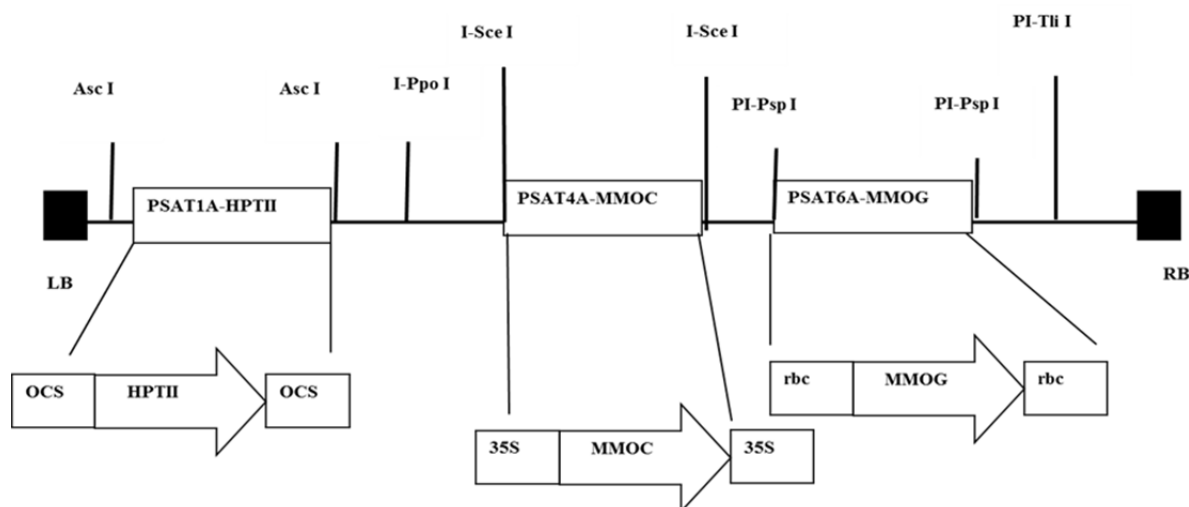


Figure 10. Construction of pRCS2-Bar-4MmoC-6MmoG. The gene for mmoC (the oxidoreductase associated with MMO) was synthesized for Arabidopsis codon bias and mmoG (a putative chaperone gene) was amplified from the methanotrophic bacterium *Methylosinus trichosporium* OB3b by PCR. The Bar gene confers resistance to phosphinothricin (PPT) .

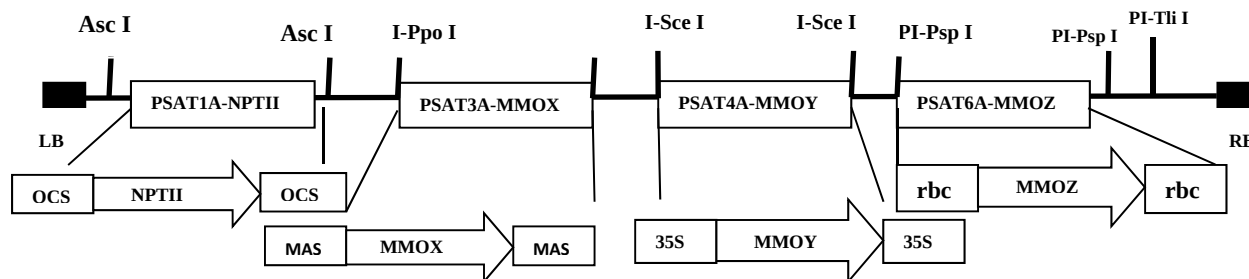


Figure 11. Construction of pRCS2-NptII-3X-4Y-6Z. The genes coding the methane monooxygenase, mmoX, mmoY, and mmoZ were synthesized with the codon sequences modified for Arabidopsis bias. The nptII gene confers kanamycin resistance.

Transformation of Arabidopsis with five sMMO genes

After two rounds of transformation and selection using two markers, seedlings resistant to both markers were selected on agar plates (Figure 12). Nineteen lines were analyzed by PCR for the presence of the nptII and bar resistance genes, the mmoX, mmoY, mmoC and mmoG genes (Figure 14). Of these eleven lines were further tested by RT-PCR for the presence of mRNA, showing expression of the genes for mmoX, mmoY, mmoZ, mmoC, and mmoG (Figure 15).

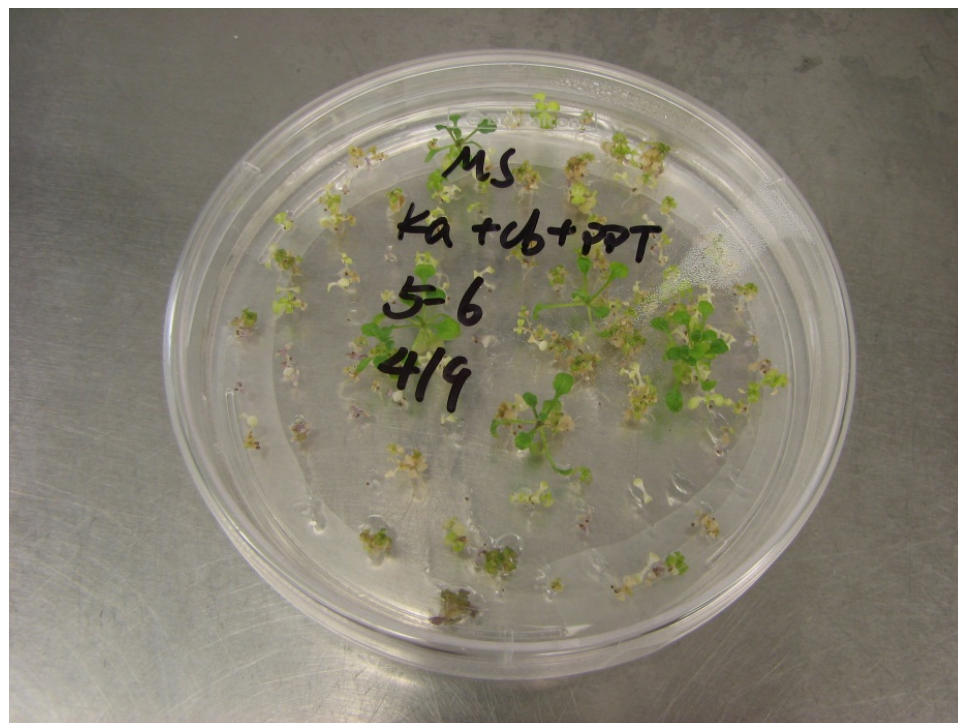


Figure 12. Screening of mmoX, mmoY, mmoZ, mmoC, and mmoG transformed Arabidopsis. Arabidopsis transformed with mmoX, mmoY, and mmoZ genes was infected with Agrobacterium EHA105 harboring prcs2-4mmoc-6mmog vector by floral dipping method. The T1 seeds were sowed on MS medium with kanamycin at 50 mg/L and PPT at 10 mg/L. Some lines with both kanamycin (Ka) and phosphinothricin

(ppt) resistance grew on the selection medium. Carbenicillin (cb) was also added to suppress the *Agrobacterium* remaining from the floral dip procedure.



Figure 13. Healthy transgenic *Arabidopsis*. Transgenic XYZ-CG *Arabidopsis thaliana* were grown and selected on MS medium containing 75mg/L kanamycin and 7.5 phosphinothricin.

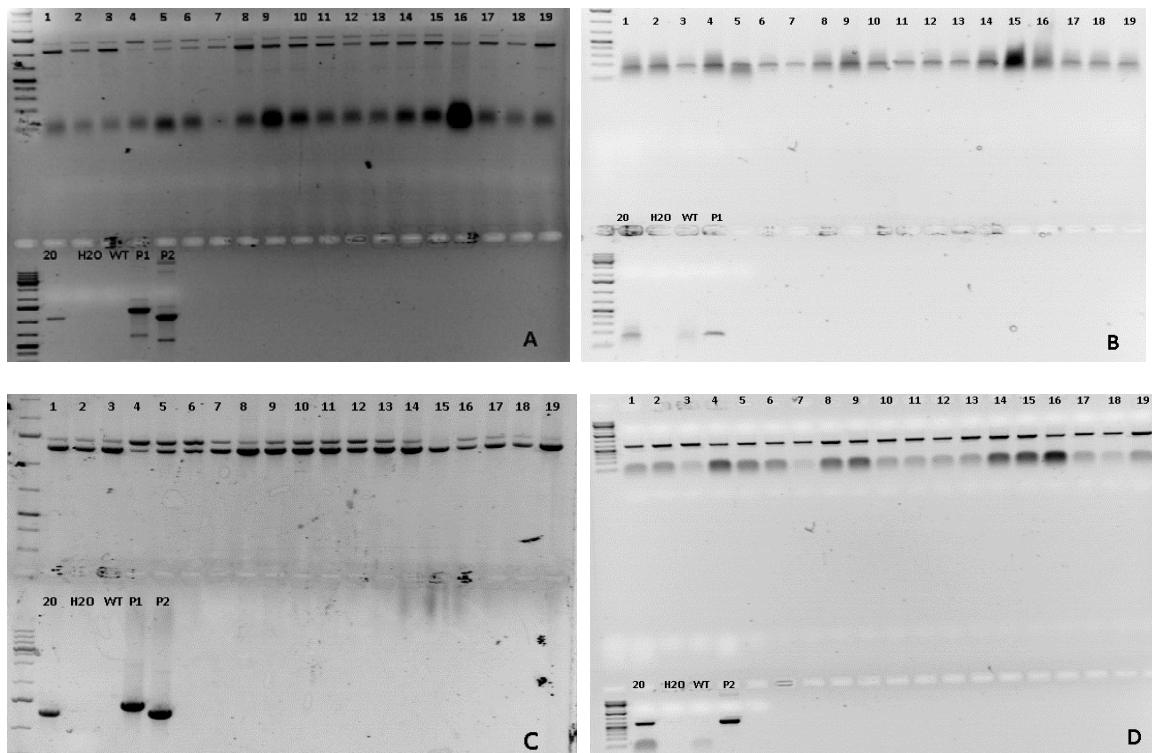


Figure 14. PCR to confirm the transformation of *Arabidopsis* with *mmoX*, *mmoY*, *mmoZ*, reductase gene *mmoC*, and chaperone gene *mmoG*. 1-20, lines of *Arabidopsis* with kanamycin and PPT resistance. (A) PCR to confirm the insertion of *nptII* and *Bar* into genome of *Arabidopsis*. (B) PCR to confirm the insertion of *mmoX*. (C) PCR to confirm the insertion of *mmoY* and *mmoC*. (D) PCR to confirm the insertion of *mmoG*. P1, vector of pRCS2-NptII-3X-4Y-6Z. P2, vector of pRCS2-Bar-4MmoC-6MmoG.

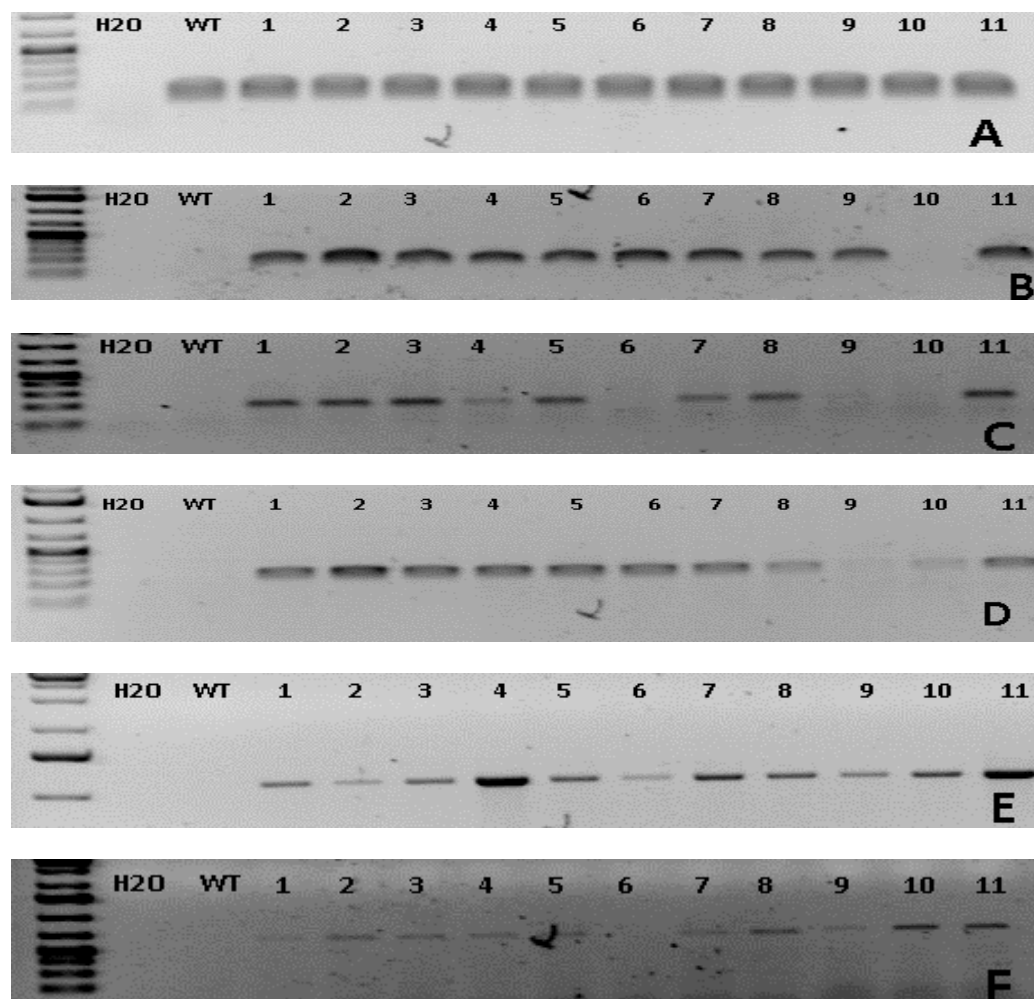


Figure 15. RT-PCR to confirm the expression of mmoX (B), mmoY (C), mmoZ (D), mmoC (E), and mmoG (F). (A) RT-PCR to confirm expression of housekeeping gene EF1a as a positive control.

Lines showing strong RT-PCR bands were selected for further testing for phenotypic expression of methane oxidation and oxidation of naphthalene to naphthol.

Analysis of phenotypic expression of sMMO

Methane uptake by *Arabidopsis thaliana* transformed with mmoXYZ

Selected *Arabidopsis* plants transformed with the genes for the three subunits of soluble methane monooxygenase, mmoX, mmoY, and mmoZ were placed in enclosed vials along with wild type *Arabidopsis* plants as controls (Figure 16). The vials were capped with Teflon lined silicon septa, through which methane was injected into the headspace of each vial using a gas-tight syringe to produce an initial concentration in the headspace of approximately 150 ppmv. Over the course of eight days the concentration of methane in the headspace was monitored by GC-FID using hand injections with a gas-tight syringe (500 μ L). A typical time course of the methane concentration is shown in Figure 17. The large error bars were due to 3 vials in which methane rapidly went to zero. We attribute those losses to leakage.



Figure 16 --Set up for analysis of methane consumption by Arabidopsis transformed with MMO genes, mmoX, Y, Z. 40 mL volatile organics analysis (VOA) vials with Teflon lined silicon septa were used to incubate whole Arabidopsis plants in approximately 10 mL media. The vials were incubated at room temperature illuminated for up to eight days

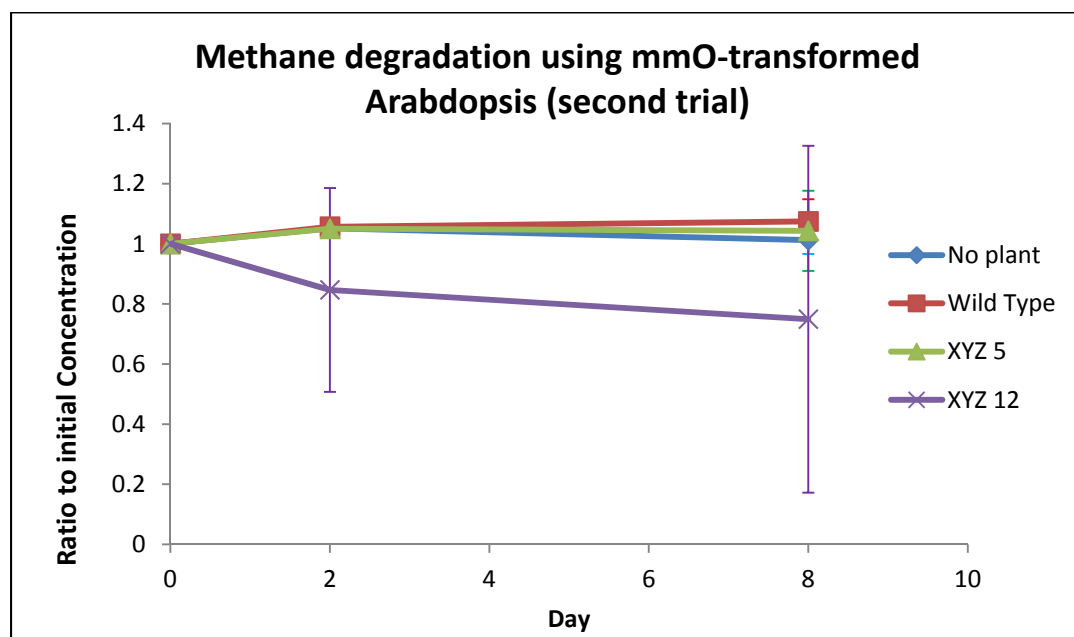


Figure 17. Methane Degradation using Transformed Arabidopsis with mmoX, mmoY, mmoZ (second trial)

Methane uptake by *Arabidopsis thaliana* transformed with mmo XYZ-CG

To reduce leaks we changed the incubation environment to 160 mL serum vials, closed with crimped, thick butyl rubber septa (Figure 18).

Healthy transgenic Arabidopsis and wild type Arabidopsis were transferred into 160 mL serum vials for methane uptake experiment. There were four replicates for each set in this experiment. Methane was

injected into each vial at 150 ppm. GC-FID was used for the methane analysis. After 7 days of the experiment, there was no significant difference in methane concentration between wild type *Arabidopsis* and transgenic *Arabidopsis* (Figure 20 and Figure 19). The large error bars indicate that there was still some problem with leakage of methane from the vials.



Figure 18. *Arabidopsis* in 160mL vials

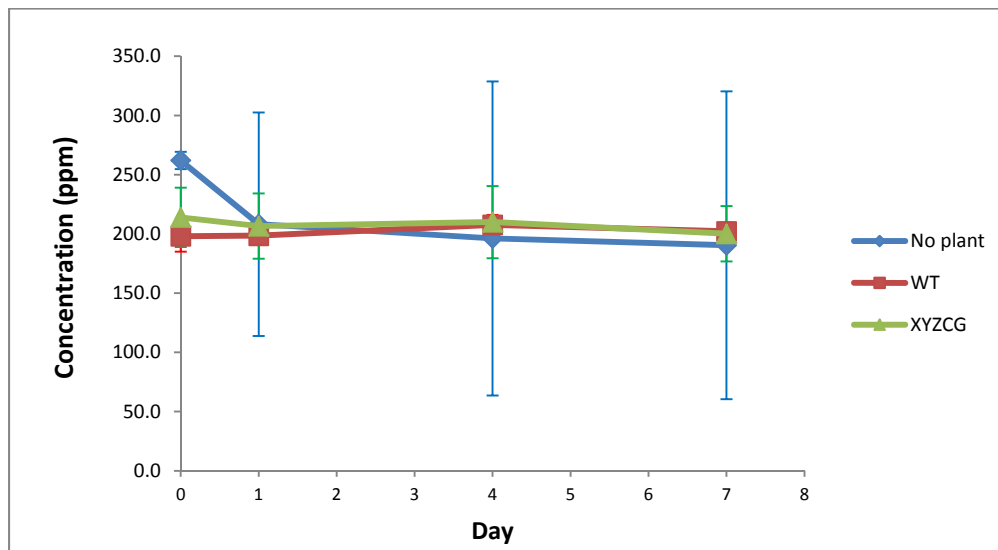


Figure 19. The time course of methane uptake by *Arabidopsis thaliana*. Methane concentrations in vials containing no plants, only media, wild type plants (WT), and transgenic plants modified with mmoXYZCG.

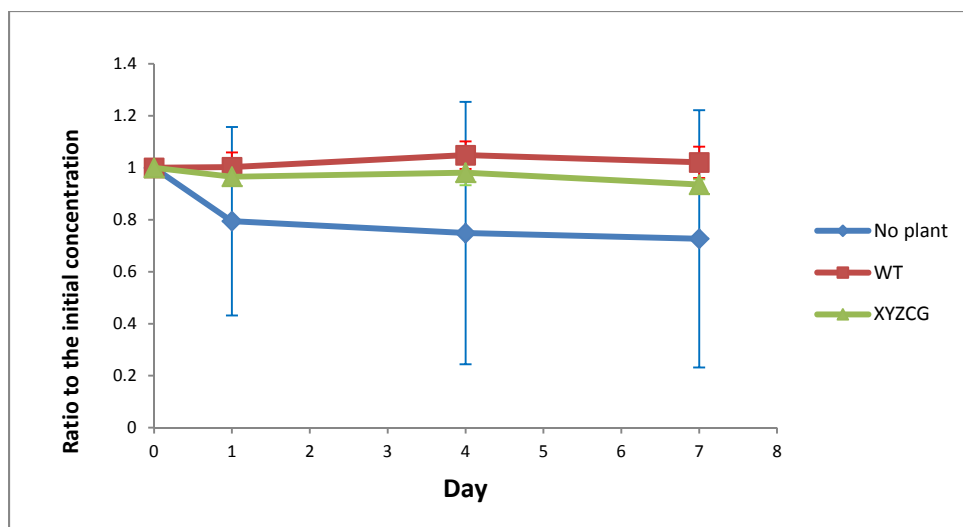


Figure 20. The time course of methane uptake by *Arabidopsis thaliana*. The ratio of the methane concentrations compared to the initial methane concentration in vials containing no plants, only media, wild type plants (WT), and transgenic plants modified with mmoXYZCG.

The methane uptake analysis was repeated three times with the mmoXYZCG and wild-type control plants. The data from these three experiments are shown plotted together in Figure 21, as the fraction remaining of the measured concentration compared to the initial concentration. Although the data do not support a clearly evident removal of methane by the mmoXYZCG transgenics, an analysis by ANOVA indicated a greater removal of methane as a function of time at the $P = 0.003$ level (F statistic = 9.44, Table 4). Thus the data support a slight removal of methane by the mmoXYZCG transgenic *Arabidopsis* plants.

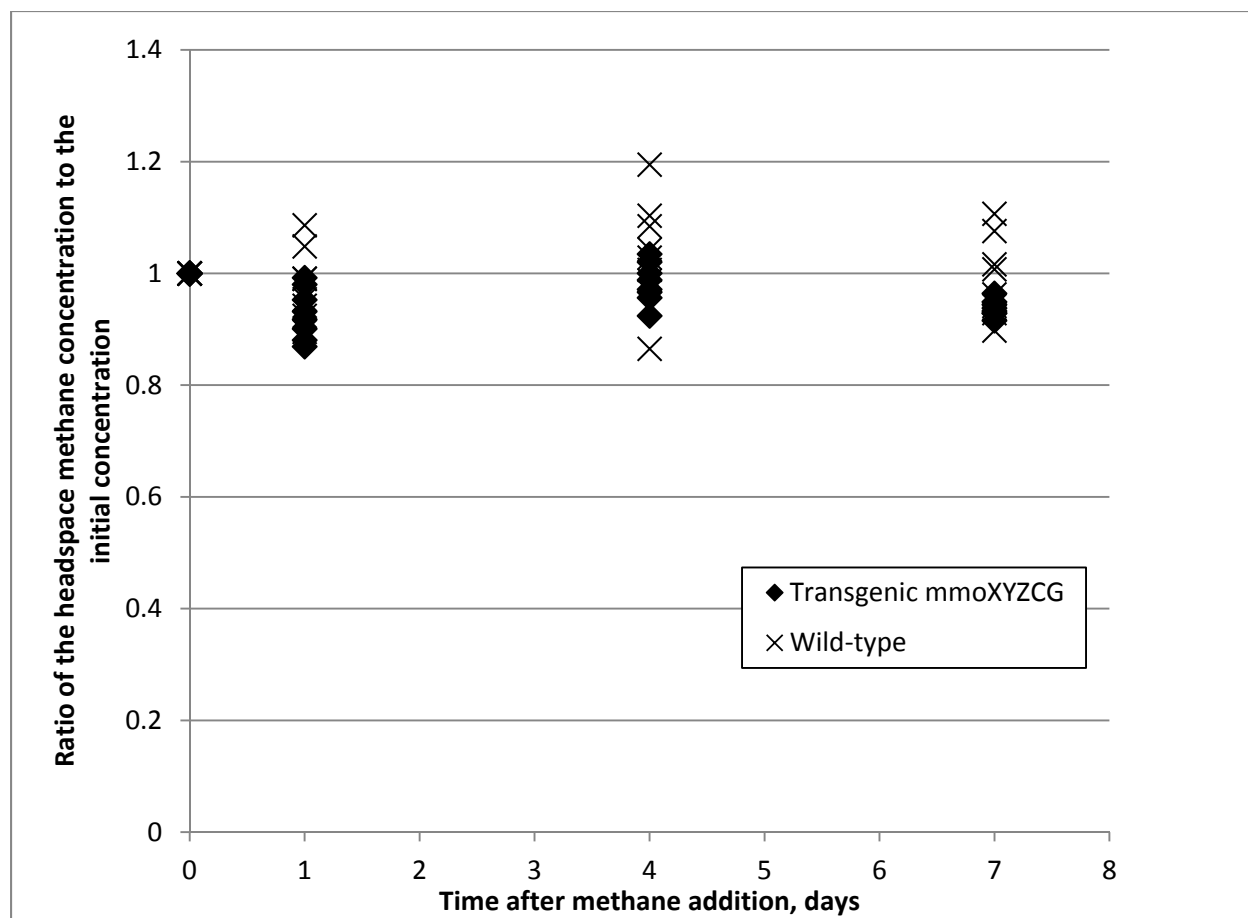


Figure 21 – Compilation of methane activity tests. Fraction remaining of headspace methane concentrations as a function of time.

Table 4 – ANOVA analysis of the methane removal time courses, as indicated by the ratios of the measured methane to the initial methane.

Anova: Two-Factor With Replication

SUMMARY	0	1	4	7	Total
<i>MMO</i>					
Count	8	8	8	8	32
Sum	8	7.424466	7.861247	7.500146	30.78586
Average	1	0.928058	0.982656	0.937518	0.962058
Variance	0	0.002006	0.001278	0.000221	0.001726
<i>WT</i>					
Count	8	8	8	8	32
Sum	8	7.83017	8.311672	7.936501	32.07834
Average	1	0.978771	1.038959	0.992063	1.002448
Variance	0	0.004079	0.00922	0.005324	0.004723
<i>Total</i>					
Count	16	16	16	16	
Sum	16	15.25464	16.17292	15.43665	
Average	1	0.953415	1.010807	0.96479	
Variance	0	0.003526	0.005744	0.003381	

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	0.026102	1	0.026102	9.436688	0.003281	4.012973
Columns	0.03627	3	0.01209	4.370991	0.00779	2.769431
Interaction	0.008766	3	0.002922	1.056397	0.374981	2.769431
Within	0.154895	56	0.002766			
Total	0.226034	63				
Sample	0.197378	2	0.098689	3.560323	0.032796	3.105157
Columns	0.091665	3	0.030555	1.102312	0.352875	2.713227
Interaction	0.083579	6	0.01393	0.502537	0.804802	2.208554
Within	2.328407	84	0.027719			
Total	2.70103	95				

Analysis of soluble MMO activity in the plants by monitoring the oxidation of naphthalene to naphthol.

The cometabolic oxidation of naphthalene to naphthol has been used as a method for confirming expression and activity of the soluble methane monooxygenase (Brusseau, Tsien et al. 1990). A colored complex is formed between 1-naphthol (formed when sMMO oxidizes naphthalene) and o-dianisidine (tetrazotized). We attempted to detect the naphthol produced by sMMO activity in the transgenic plants, but plant pigments interfered with the detection of the colored product.

Alternatively we measured the production of 1-naphthol using GC-MS. We cultured 0.8 g wild-type and mmoXYZ- or mmoXYZCG-transformed Arabidopsis in 50 mL ½ MS medium with saturated naphthalene in flasks closed with sponge and foil. The plants were cultured at 20°C with shaking for 4 days under natural daylight illumination. The plant tissue was collected, ground in a mortar and pestle, and suspended in 40 mL pH 5 acetate buffer. Centrifuge at 4,000 rpm for 20 min at 4°C to pellet the ruptured plant materials. The supernatant was hydrolyzed with 25 µL of β-glucuronidase/arylsulfatase at 37°C for 20 hours.

Plant tissue extraction was followed by sample cleanup using an AutoTrace SPE workstation (Caliper life sciences). The SPE column (Hypersep retain PEP column) was pre-activated with 6 mL of methanol and 8 mL distilled water. The hydrolyzed sample (20 mL) was loaded onto SPE column and slowly aspirated. The column was washed with 6 mL of distilled water and gently dried by aspiration for 30 min. The column was eluted with 6 mL methanol and the methanol eluent saved for derivatization and analysis.

The methanol extract obtained in the SPE-procedure was evaporated to dryness under a nitrogen stream and the residue silylated with N,O-bis (trimethylsilyl) acetamide and pyridine. The reaction mixture was heated at 60°C for 1 hour and after cooling to room temperature was used for analysis by GC-MS.

GC-MS analysis was performed using a Shimadzu GCMS-QP2010 plus with a split/splitless injector. The analytical column was a SHRXI-5MS. The oven temperature program began at 130 °C, was held for 1min, raised to 200 °C at 3°C/min, raised to 270 °C at 10°C/min and held for 10 min. The ions selected in this study were m/z 185, 201, 216, which are analytical for TMS-1-naphthol.

More naphthol was produced in the media of mmoXYZ transformed plants than in the wild-type as shown in Figure 22. The difference in production of naphthol between the transformed and wild type or Arabidopsis was greater when naphthol was extracted from plant tissues (Figure 23). When Arabidopsis plants transformed with mmoXYZCG were tested the apparent MMO activity, as indicated by naphthol production in the plant tissues, was further increased (Figure 24). These results were repeated in a subsequent experiment with the same result: significantly increase naphthol production in the mmoXYZCG transformed plants compared to wild-type as shown in Figure 25.

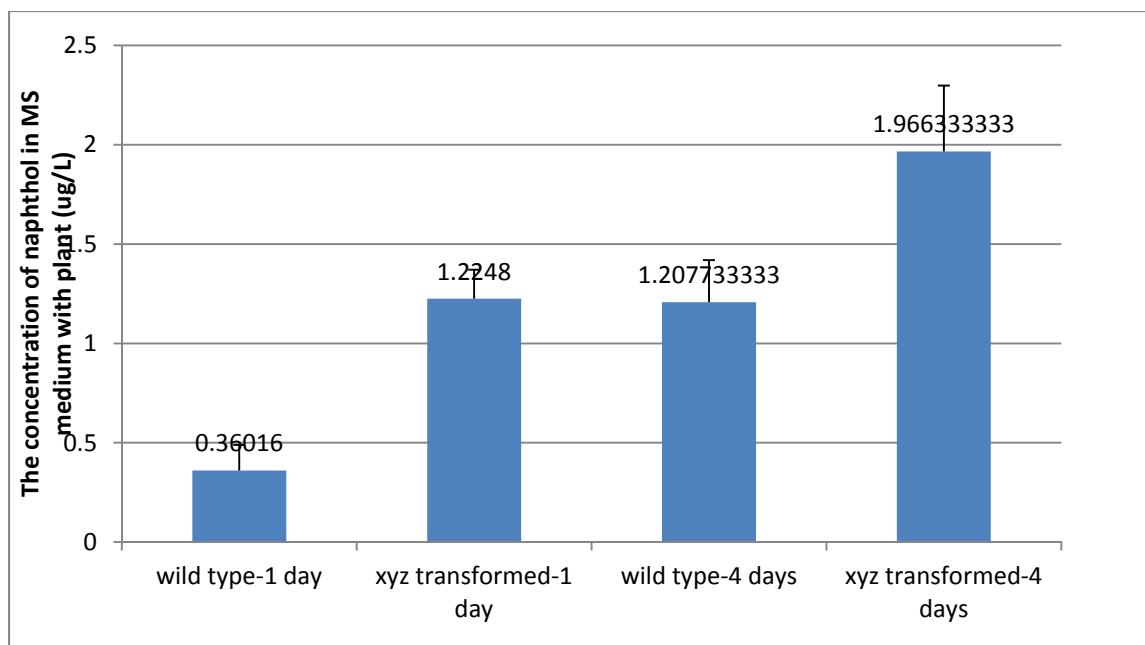


Figure 22. Production of 1-naphthol in the media of Arabidopsis wild type and mmoXYZ-transformed plants incubated with naphthalene. The wild type and mmoXYZ transformed plants were cultured in liquid MS medium with saturated naphthalene and cultured with shaking. The medium was sampled after 1 day or 4 days culture and the naphthol concentration was analyzed by GC- MS.

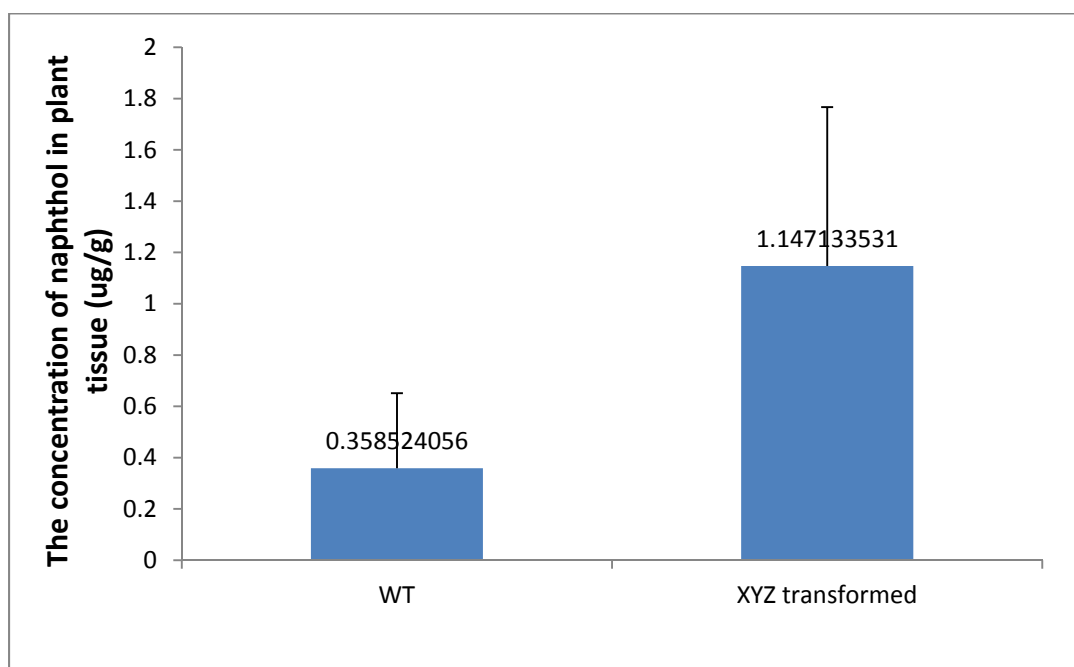


Figure 23. Production of 1-naphthol in the tissues of Arabidopsis wild type and mmoXYZ-transformed plants incubated with naphthalene. The wild type and mmoXYZ transformed plants were cultured in liquid MS medium with saturated naphthalene with shaking for 7 days. The plant material was sampled and naphthol was extracted from plant tissue and analyzed by GC-MS.

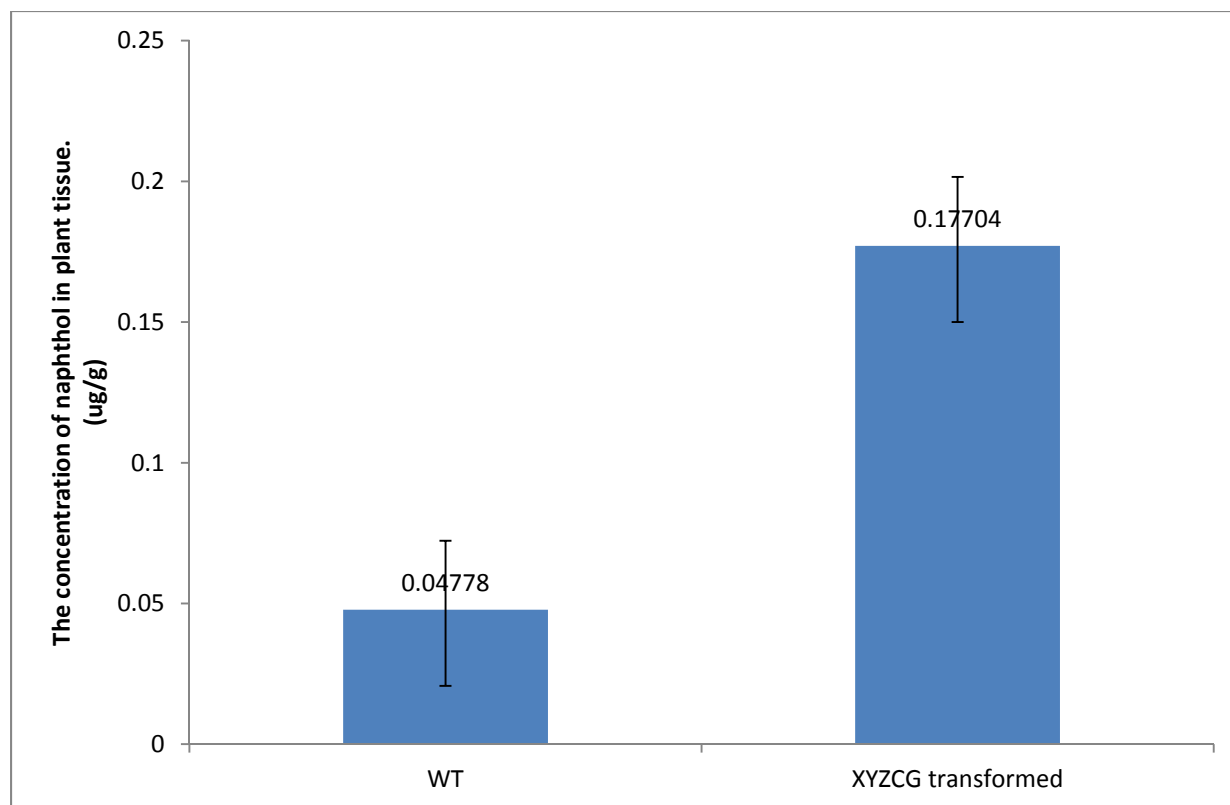


Figure 24. Detection of increased naphthol production from naphthalene in the tissue of wild-type and mmoXYZCG-transformed *Arabidopsis*. The plants were about 400 mg wet biomass and were 25-day old at the start of the experiment. The plants were incubated in capped 20 mL liquid MS medium with saturated naphthalene and cultured with shaking for 7 days. The plant material was sampled and the concentration of naphthol in plant was analyzed by GC-MS.

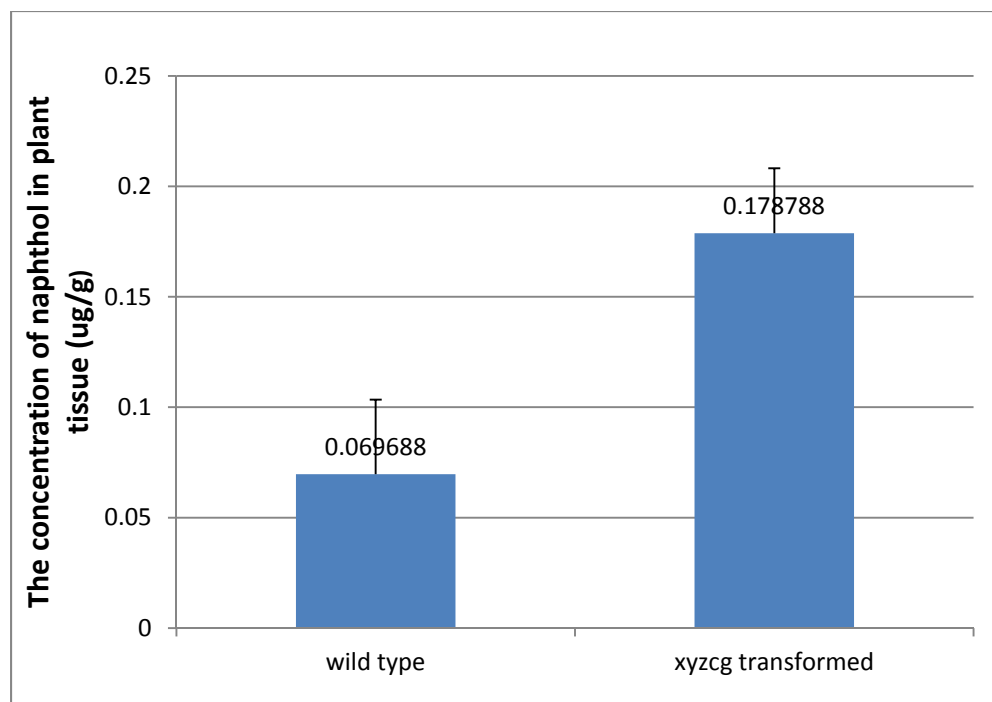


Figure 25 -- The production of naphthol from naphthalene in tissues of Arabidopsis plants transformed with mmoXYZCG genes compared to wild-type. Six plants of each type were incubated in enclosed vessels with naphthalene with shaking and daylight illumination for 7 days.

Taken together these data provide support for the proposition that the mmoXYZCG transformed plants expressed an active methane monooxygenase, albeit that the activity was slight.

Discussion

The plants transformed with mmoXYZCG demonstrated a slight, but significant increase in removal of methane and 2-4 fold increase in naphthol production from naphthalene compared to untransformed wild-type Arabidopsis plants. The former assay is a classic determinant of sMMO activity. Thus, our data provide support for functional activity of sMMO in our transformed plants. Although the activity was small, these data suggest that the MMOH was properly assembled.

The lack of the gene for Protein B, mmoB, in our constructs was unfortunate. Recent studies have shown conclusively the role of mmoB in maximizing throughput of methane and oxygen during the operation of the MMOH (Zhang and Lipscomb 2006; Mitic, Schwartz et al. 2008; Lee, McCormick et al. 2013). In the presence of mmoB, oxidation of methane and surrogate substrates is increased by orders of magnitude (Liu, Nesheim et al. 1995). The product of mmoB, Protein B aka MMOB, is associated 2:1 with the MMOH complex. MMOB functions to open channels for access to the diiron active site by methane and oxygen and allows egress of the product, and methanol from the interior of the MMOH:MMOB complex (Whittington, Rosenzweig et al. 2001; Lee, McCormick et al. 2013).

Ongoing work

We are continuing to work on the following aspects specific to our goals:

1. Western blots of the protein extract of mmoXYZCG, using the antibodies derived from mmoX and mmoZ and commercial antibodies for the FLAG tag for mmoY. We expect these assays to provide additional evidence for expression of these proteins in Arabidopsis.
2. Assay of sMMO activity in the mmoXYZCG transformed plants using propylene oxide production from propylene, a second classic assay for sMMO activity.
3. Inhibition of propylene or naphthalene oxidation by addition of methane. This experiment will confirm the role as substrate of methane for the enzymes responsible for co-oxidation of naphthalene or propylene.
4. Construction of vectors for transformation of the mmoXYZCG transformed lines with mmoB. The PSAT system will be used again, this time with hygromycin resistance gene, hptI, as the selection marker. We expect that the resultant transformed plants, mmoXYZCGB, will have greatly increased sMMO activity.

Conclusions

We were able to fulfill the primary objective of the proposal; the transformation of plants with genes essential for the activity of the soluble methane monooxygenase of *M. trichosporium*. PCR and RT-PCR for the genes of the MMOH subunits (mmoX, mmoY, and mmoZ for the α , β , and γ subunits respectively), the mmoC gene for the MMOC reductase, and the gene for the putative helper protein, mmoG. Attempts to transiently transform or to simultaneously transform with separate binary vectors for the xyz subunit genes were unsuccessful. We also abandoned objective 2, the development of vectors for chloroplast transformation with the mmo operon.

Construction of vectors for nuclear transformation using the PSAT system incorporated mmoX, mmoY, and mmoZ genes in the first vector with kanamycin resistance gene as the selection marker. After transformation and selection, several plant lines were selected which incorporated the three genes for the subunits of MMOH, the hydroxylase. These plants, designated mmoXYZ, demonstrated increased oxidation of naphthalene to naphthol.

The mmoXYZ transformed plants were transformed again with a second vector constructed with the mmoC gene for the reductase and mmoG for a putative helper protein with bar gene for resistance to the second selection marker, ppt. Due to technical problems with its synthesis, the mmoB gene for the regulatory protein, Protein B, was not included. The plants resulting from the second transformation were confirmed by PCR and RT-PCR to have the genes of interest and to express their mRNA.

The plants transformed with mmoXYZCG demonstrated a slight, but significant increase in removal of methane and 2-4 fold increase in naphthol production from naphthalene compared to untransformed wild-type Arabidopsis plants. The former assay is a classic determinant of sMMO activity. Thus, our data provide support for functional activity of sMMO in our transformed plants. Although the activity was small, these data suggest that the sMMO was properly assembled and functional in plants. The results

are promising for greatly increased methane oxidizing activity when the MMOB can be expressed in our transformants.

References

- Brusseau, G. A., H.-C. Tsien, et al. (1990). "Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity." Biodegradation **1**: 19-29.
- Campbell, G. S. and J. M. Norman (1998). An Introduction to Environmental Biophysics. New York, Springer.
- Clough, S. J. and A. F. Bent (1998). "Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana." Plant Journal **16**(6): 735-743.
- Einhauser, A. and A. Jungbauer (2001). "The FLAG (TM) peptide, a versatile fusion tag for the purification of recombinant proteins." Journal of Biochemical and Biophysical Methods **49**(1-3): 455-465.
- Gou, Z. X., X. H. Xing, et al. (2006). "Functional expression of the particulate methane mono-oxygenase gene in recombinant Rhodococcus erythropolis." Fems Microbiology Letters **263**(2): 136-141.
- Houweling, S., T. Kaminski, et al. (1999). "Inverse modeling of methane sources and sinks using the adjoint of a global transport model." Journal of Geophysical Research-Atmospheres **104**(D21): 26137-26160.
- Jahng, D., C. S. Kim, et al. (1996). "Optimization of trichloroethylene degradation using soluble methane monooxygenase of Methylosinus trichosporium OB3b expressed in recombinant bacteria." Biotechnology and Bioengineering **51**(3): 349-359.
- Jahng, D. J. and T. K. Wood (1994). "TRICHLOROETHYLENE AND CHLOROFORM DEGRADATION BY A RECOMBINANT PSEUDOMONAD EXPRESSING SOLUBLE METHANE MONOOXYGENASE FROM METHYLOSINUS-TRICHOSPORIUM OB3B." Applied and Environmental Microbiology **60**(7): 2473-2482.
- Kristjansson, J. K. and T. C. Hollocher (1980). "1st practical assay for soluble nitrous-oxide reductase of denitrifying bacteria and a partial kinetic characterization." Journal of Biological Chemistry **255**(2): 704-707.
- Lee, S. J., M. S. McCormick, et al. (2013). "Control of substrate access to the active site in methane monooxygenase." Nature **494**(7437): 380-384.
- Liu, Y., J. C. Nesheim, et al. (1995). "Gating Effects Of Component B On Oxygen Activation By the Methane Monooxygenase Hydroxylase Component." Journal of Biological Chemistry **270**(42): 24662-24665.
- Lontoh, S. and J. D. Semrau (1998). "Methane and trichloroethylene degradation by Methylosinus trichosporium OB3b expressing particulate methane monooxygenase." Applied and Environmental Microbiology **64**(3): 1106-1114.
- Massman, W. J. (1998). "A review of the molecular diffusivities of H₂O, CO₂, CH₄, CO, O₃, SO₂, NH₃, N₂O, NO, AND NO₂ in air, O₂ AND N₂ near STP." Atmospheric Environment **32**(6): 1111-1127.
- Merkx, M., D. A. Kopp, et al. (2001). "Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: A tale of two irons and three proteins." Angewandte Chemie-International Edition **40**(15): 2782-2807.
- Mitic, N., J. K. Schwartz, et al. (2008). "CD and MCD studies of the effects of component B variant binding on the biferrrous active site of methane monooxygenase." Biochemistry **47**(32): 8386-8397.
- Rylott, E. L., R. G. Jackson, et al. (2006). "An explosive-degrading cytochrome P450 activity and its targeted application for the phytoremediation of RDX." Nature Biotechnology **24**(2): 216-219.

- Sainsbury, F., E. C. Thuenemann, et al. (2009). "pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants." Plant Biotechnology Journal **7**(7): 682-693.
- Tzfira, T., G. W. Tian, et al. (2005). "pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants." Plant Molecular Biology **57**(4): 503-516.
- Whittington, D. A., A. C. Rosenzweig, et al. (2001). "Xenon and halogenated alkanes track putative substrate binding cavities in the soluble methane monooxygenase hydroxylase." Biochemistry **40**(12): 3476-3482.
- Wood, T. K. (2002). "Active expression of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b in heterologous hosts." Microbiology-Sgm **148**: 3328-3329.
- Zhang, J. Y. and J. D. Lipscomb (2006). "Role of the C-terminal region of the B component of *Methylosinus trichosporium* OB3b methane monooxygenase in the regulation of oxygen activation." Biochemistry **45**(5): 1459-1469.

Appendix

Sequences of the subunits and helper genes of the soluble methane monooxygenase of *Methylosinus trichosporium* OB3b, as optimized for plant codon usage to increase expression and translation in plants.

MMOX :

ATGGCTATCTCTCTCGCTACTAAGGCTGCTACTGATGCTCTTAAGGTAAACAGAGCACCTGTTGGAGTTGAACCTCA
AGAGGTTACACAAGTGGCTTCAATCTTTCAACTGGGATTTCAAAGAGAACAGGACCAAGTACCCTACCAAGTATCATA
TGGCTAACGAGACAAAAGAGCAGTTCAAGGTTATCGCTAAAGAATACGCTAGGATGGAAGCTGCTAAGGATGAGAGA
CAATTCGGAACCTTTCTCGATGGACTTACTAGACTTGGAGCTGGAAACAAGGTTACCCTAGATGGGGAGAACTAT
GAAGGTGATCTCTAACTTCCTTGAGGTGGGAGAGTACAATGCTATTGCTGCTTCTGCTATGCTCTGGGATTCTGCTA
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AACCCTACTACTCTAAGCACTACCATGATCCTGCTGGACATAACGATGCTAGAAGAACTAGAGCTATCGGACCTCT
TTGGAAGGGAATGAAGAGGGTTTTTCGCTGATGGATTCATCTCTGGTGATGCTGTTGAGTGCTCAGTTAACTTGCAAC
TTGTTGGAGAGGCTTGCTTCACTAACCTCTTATCGTTGCTGTTACTGAATGGGCTTCTGCTAACGGTGATGAGATT
ACTCCTACTGTTTTCTCTCTGTGGAAACCGATGAGCTTAGACATATGGCTAATGGATACCAGACCGTTGTGTCTAT
TGCTAATGATCCAGCTTCTGCTAAGTTCTCAACACTGATCTCAACAACGCTTTCTGGACTCAGCAGAAGTACTTCA
CTCCTGTTCTTGGATACCTCTTCGAGTACGGATCTAAGTTTAAGGTTGAGCCTTGGGTAAAGACCTGGAATAGATGG
GTTTACGAAGATTGGGGAGGAATTTGGATCGGTAGACTTGGAAGTACGGTGTTGAATCTCCTGCTTCTCTCAGAGA
TGCTAAGAGAGATGCTTACTGGGCTCATCATGATCTTGCTCTTGCTGCTTACGCTATGTGGCCTCTTGGATTTGCTA
GACTTGCTTTGCCTGATGAAGAAGATCAGGCTTGTTTTGAAGCTAATTATCCTGGATGGGCTGATCACTACGGAAAG
ATTTTTAACGAGTGGAAGAAGCTCGGATACGAAGATCCTAAGTCTGGATTCATCCCTTACCAATGGCTTTTGGCTAA
CGGACATGATGTGTACATCGATAGAGTGTCTCAGGTTCCATTCATCCCATCTCTTGCTAAGGGAACCTGGATCTCTTA
GAGTGCATGAGTTCAACGGAAAGAAACACTCTCTCACTGATGATTGGGGTGAGAGACAATGGCTTATTGAACCTGAG
AGATACGAGTGCCATAACGTTTTCGAACAGTATGAGGGAAGGGAACCTCTCTGAAGTTATTGCTGAAGGACATGGTGT
GAGGTCTGATGGAAAGACTCTTATTGCTCAGCCTCACACTAGAGGTGATAACCTTTGGACTCTTGAGGATATTAAGA
GGGCTGGATGCGTTTTCCCTGATCCTCTTGCTAAGTTCTGA

MMOY :

ATGTCTCAGCCTCAGTCATCTCAGGTTACAAAGAGAGGACTTACCGATCCTGAGAGAGCTGCTATTATTGCTGCTGC
TGTTCTGATCATGCTCTTGATACTCAGAGGAAGTACCACTACTTCATCCAACCTAGATGGAAGCCACTTTCTGAGT
ACGAGCAGCTTTCTTGTTACGCTCAACCTAACCTGATTGGATTGCTGGTGGACTTGATTGGGGAGATTGGACTCAA
AAGTTCCATGGTGGTAGACCATCTTGGGGAAATGAGTCTACTGAGCTTAGAACCACCGATTGGTACAGACATAGAGA
TCCTGCTAGAAGATGGCATCATCCATACGTGAAGGATAAGTCTGAAGAGGCTAGATACACCCAGAGATTTCTTGCTG
CTTACTCTTCTGAGGGATCTATCAGAACCATCGATCCTTATTGGAGGGATGAGATCCTCAACAAGTACTTTGGTGCT
TTGCTCTACTCTGAGTACGGACTTTTCAACGCTCATTCTTCTGTGGGAAGAGATTGCCTCTCTGATACAATCAGACA
GACTGCTGTTTTGCTGCTCTCGATAAGGTTGACAACGCTCAAATGATCCAGATGGAAAGGCTCTTTATCGCTAAGT
TGGTGCCTGGATTGATGCTTCTACTGATGTGCCTAAGAAGATTTGGACCACCGATCCTATCTACTCTGGTGCTAGA
GCTACTGTTCAAGAGATTTGGCAAGGTGTTCAAGATTGGAACGAGATTCTTTGGGCTGGACATGCTGTTATGATCGC
TACTTTTGGACAGTTGCTAGAAGAGAGTTCTTCCAAAGACTCGCTACTGTGTACGGTGATACTCTTACTCCTTTCT
TCACCGCTCAATCTCAGACTTACTTCCAGACTACTAGGGGTGCTATCGATGATCTTTTCGTTTACTGCCTCGCTAAC
GATTCTGAGTTTGGTGCTCACAACAGGACATTTCTTAACGCTTGGACTGAGCATTACCTCGCTTCATCTGTTGCTGC
TCTCAAGGATTTGTTGGACTTTATGCTAAGGTTGAGAAGTCTAGGGCTGATAGATCAAGAAGAAGATTGAGGGGAG
CTGCTGCTTCTTCTGCTATTGGAAGATCAATCACCCCTGATAAGATCGGATTCAGAGTTGATGTGGATCAGAAAGTT
GATGCTGTTCTCGCTGGATACAAGAAGTGA

MMOZ :

ATGGCTAAGAGAGAGCCTATCCACGATAACTCTATCAGAACTGAATGGGAGGCTAAGATCGCTAAGCTCACTTCTGT
TGATCAGGCTACTAAGTTCATCCAGGATTTGAGACTCGCTTACACCTCTCCATTGAGAAAGTCTTACGATATCGATG
TCGATTACCAGTACATCGAGAGGAAGATCGAAGAGAAGCTCTCTGTTCTCAAGACTGAGAACTCCCTGTTGCTGAT
CTCATCTAAGGCTACTACTGGTGAAGATAGAGCTGCTGTTGAGGCTACTTGGATTGCTAAGATTAAGGCTGCTAA
GTCTAAGTACGAGGCTGATGGAATCCACATCGAGTTCAGACAACCTTTACAAGCCTCCTGTTCTCCCTGTTAACGTTT
TCCTTAGAACTGATGCTGCTCTCGGAACTGTTCTCATGGAAATCAGAAACACCGATTACTACGGAACCTCCTTTGAG
GGACTTAGAAAAGAACCTGGTGTTAAGGTTCTCCATCTCCAGGCTTGA

MMOB:

ATGTCATCTGCTCACAACGCTTACAACGCTGGAATCATGCAAAGACTGGAAAGGCTTTCGCTGATGAGTTTTTCGC
TGAAGAAAACAGGTGGTGCATGAATCTAACGCTGTTGTTCTCGTGCTCATGAAGTCTGATGAGATCGATGCTATCA
TCGAGGATATCGTTCTCAAGGGTGGAAAGGCTAAGAACCCTTCTATCGTTGTTGAGGATAAGGCTGGATTCTGGTGG
ATTAAGGCTGATGGTGCATCGAAATTGATGCTGCTGAAGCTGGTGAGCTTCTTGAAAGCCTTCTCTGTTTACGA
TCTCCTCATCAACGTGTCATCTACTGTTGGTAGAGCTTACACTCTCGGAACTAAGTTCACTATCACCTCTGAGCTTA
TGGGACTTGATAGGGCTCTCACTGATATCTGA

MMOC:

ATGTACCAGATCGTGATCGAGACTGAAGATGGTGAGACTTGTAGAAGAATGAGGCCTTCTGAGGATTGGATTTCAAG
AGCTGAAGCTGAGAGAAACCTTCTTGCTTCTTGTAGAGCTGGATGTGCTACTTGTAAGGCTGATTGCACTGATGGT
ATTACGAGCTTATCGATGTTAAGGTTCAAGGCTGTTCTCTCTGATGAAGAGGAAGATGGAAAGGTTTTGCTCTGCAGA
ACCTTCCCTAGAAGTGATCTTCATCTCTCGTGCCTTACACCTACGATAGAATTTCTTTCGAGGCTATCCAGACTAA
CTGGCTTGCTGAAATCCTTGCTTGCGATAGGGTGTCTAACGTTGTGAGACTTGTTCTTCAGAGGTCAAGACCTA
TGGCTGCTAGAATCTCTCTCAATTTGTTCTTGACAGTTCGTGGATATTGAGATTCCTGGAACCTCACACTAGAAGG
TCTTACTCTATGGCTTCTGTTGCTGAGGATGGACAACTTGAGTTTATCATCAGACTCTTGCTGATGGTGCTTTCTC
TAAGTTCCTTCAAACCTGAGGCTAAGGTGGGAATGAGAGTTGATCTTAGAGGACCTGCTGGATCTTCTTCTTCATG
ATCACGGTGGAAAGGTCTAGGGTTTTCGTTGCTGGTGGAACCTGGACTTTCTCTGTTCTTCTATGATCAGACAGCTC
GGAAAGGCTTCTGATCCTTCTCTGCTACTCTTTTGTTGCGAGTTACCAACAGGGAAGAGTTGTTCTATGTGGATGA
GCTTAAGACTCTCGCTCAGTCTATGCCTACTCTTGAGTTAGAATCGCTGTGGTTAACGATGATGGTGGAAACGGTG
TTGATAAGGGAACCTGTTATCGATCTTCTCAGAGCTGAGCTTGAGATTGATCTTTTGCTTGACATGCTAGGAGACGT
AGACGTAGAGAACTGCTAGGTCTTGACAGAGAGGATCATAGAGATAGATGTCCTGCTTGAGGTCTGATTTCTTGA
AAAGTTCCTCGCTTCTGGATGA

MMOG: This sequence was designed by us for optimal activity in plants, but the synthesis was delayed. Thus we used the sequence amplified from a methanotrophs. We include the plant optimized sequence for completeness.

ATGG ACTACAAGGA TGACGATGAC AAGATGACTA ATCCTAGAAA GAGAGAAAGA

AGAAGACCTG CTTTTGATGT TACTAGAGAA AAGTTTGTTG CTAGAAATAT TAGATTTGGA
 GATGTTGTTA GAAGGGATCT TCTTGCTGGA GTTGATGCTC TTGCTGATGC TGTTGCTGTT
 ACTCTTGGAC CTAGAGGAAG AAATGTTGTT ATTGAACATA GAGCTGCTGG ACTTCCTCCT
 GTTGCTACTA AGGATGGAGT TACTGTTGCT CAAGCTGTTG AACTTGCTGG AAGAACTCAA
 TCTGTTGGAG TTTCTCTTGT TAGACAAATG GCTACTGCTG TTGCTAAGGA AGCTGGAGAT
 GGAACACTA CTTCTGTTGT TCTTGCTAGA AGACTTGCTG CTGAAACTAG AAAGGCTCTT
 GCTGCTGGAA TGAATCCTAG AGATATTGTT CTTGGAATGG AAAAGGCTGC TAGAATTGTT
 GATAGGGATC TTGCTGCTAG AGCTAGAAGA TGTGATGATA CTAGAGCTCT TGCTCATGTT
 GCTACTCTTG CTGCTGGAGG AGATGAATCT ATTGGAGCTA TTGTTGCTGA TGCTCTTACT
 AGAGCTGGAG AAGGAGGAGT TGTTGATGTT GAACTTGGAG CTGCTCTTTG TGATGAAATG
 GATATTGTTG AAGGAATGAG ATGGGAACAA GGATATAGAT CACCTTATTT TATGACTGAT
 TCTGCTAGAA AGATTGCTGA ACTTGAAAAT CTTATATTC TTATTTATGA TAGAGTTATT
 AATCAATTTT CTGAACTTGT TCCTGCTCTT GAACTTGTTA GAAGACAAAG AGGATCTCTT
 CTTATTGTTG CTGAAAATAT TGTTGAAGAA GCTCTTCCTG GACTTCTTCT TAATCATATT
 AGAAAGAATC TTTGTTCTAT TGCTGTAAAG GGACCTGGAT ATGGAGATTC AAGATATGAA
 TTTCTTCATG ATCTTGCTGC TCTTACTGGA GGAAGAGCTA TTATGGAGGC TTGTGGAGAA
 GAACTTTCTA ATGTTACTAT GGCTCATCTT GGAAGAGCTA AGAGAGTTGT TGTTAGAGAA
 GATGATACTG TTGTTATTGG AGGAGAAGGA GATGGAGCTG CTATTACTGA AAGACTTGCT
 GCTGCTAGAC AACAAGCTGA TTGGATTACT GATGGAGATC CTTCTAAGGG ATCTCCTTCT
 GGAAAGAGAC ATGATCTTGA AAATCTTCAA ACTAGAATTA AGGCTCTTTC TGGAAAGGTT
 GTTACTATTA AGGCTGGAGG ACTTTCTGAT ATTCTTATTA AGGAAAGAAT GCAAAGAATT
 GAAAATGCTC TTGCTTCTGC TAGAGCTGCT AGGTCTGATG GAGTTGTTGC TGGAGGAGGA
 GTTGGACTTT ATAGAGCTAG AGCTGCTCTT ACTGAAGCTA CTGGAGATAC TCTTGATCAA
 ACTTATGGAA TTGCTATTGT TAGAGCTGCT CTTGATGAAC CTATTAGAAG AATTGCTGCT
 AATGCTGGAA GAGATGCTCA TGAATTTCTT TTTGAACTTA AGAGGTCTAA TGATGATTTT
 TGGGAATGG ATATGAGATC AGGAGAATGT GGTGATCTTT ATGCTGCTGG AGTTATTGAT
 CCTGCTAGAG TTAGTAGACT TGCTCTTAGA AATGCTGTTG CTAAGCTTTC TTCTCTTATG
 ACTGTTGAAT GTGCTGTTAC TCATATTCCT CTTCTGATC CTAATTATGG ATTTGATCCT

CATCTTGCTG CTGCTACTAG AGAAGATCCT AGATCATGA

MMOG: This is the MMOG sequence of the methanotroph, *Methylosinus trichosporium* OB3b. We cloned this gene and used it to transform *Arabidopsis*.

ATGACAAATCCAAGAAAACGTGAGCGGCGACGACCGGCGTTTCGACGTGACGAGGGAGAAA
TTCGTGGCTAGAAATATTCGATTCGGAGACGTCGTGCGTCGTGATCTGCTCGCCGGCGTC
GATGCGCTCGCCGACGCCGTCGCCGTCACGCTCGGCCGCGCGGTCGCAATGTGGTGATC
GAGCATCGCGCCGCGGGGCTTCCGCCCCTCGCCACCAAGGATGGCGTGACCGTCGCGCAG
GCGGTCGAGCTCGCGGGCCGCGACGCAGAGCGTCGGCGTGTGCTGGTGCGGCAGATGGCG
ACGGCCGTCGCCAAGGAGGCCGGCGACGGCACGACCACATCCGTCGTGCTGGCGCGGCGC
CTCGCCGCCGAGACGCGCAAGGCGCTCGCCGCCGGCATGAATCCGCGCGACATCGTGCTC
GGCATGGAGAAAGCGGCGCGCATCGTCGATCGTGATCTCGCCGCGCGCGCACGGCGCTGC
GACGATACACGCGCGCTCGCGCATGTGCGGACGCTGGCGGGGGCGGCGACGAGTCGATC
GGCGCAATCGTCGAGACGCCTTGACGCGCGCCGGCGAAGGCGGCGTCGTGATGTGAG
CTCGGCGCCGCTCTTTGCGACGAGATGGACATCGTCGAAGGCATGCGTTGGGAGCAGGGC
TATCGATCGCCTTATTTTCATGACCGACAGCGCACGCAAGATCGCCGAGCTCGAGAATCCC
TATATTCTGATCTATGACCGCGTCATCAATCAATTCTCCGAGCTGGTGCCGGCGCTGGAG
CTGGTGCGCCGTCAGCGCGGCAGCCTGCTGATCGTCGCCGAGAACATCGTCGAGGAGGCT
CTGCCCCGGCCTCTTGCTCAATCATATCCGCAAGAATCTCTGCTCGATCGCGGTGAAGGGT
CCTGGTTATGGCGACAGCCGCTATGAGTTCTGACGATCTCGCGGCGCTGACCGGCGGA
CGCGCGATCATGGAAGCCTGCGGAGAAGAATTGTCCAATGTGACGATGGCGCATCTCGGC
CGCGCGAAACGCGTCGTGTCGTGCGCGAGGATGATACGGTGGTGATCGGCGGCGAGGGCGAC
GGCGCGGCGATCACTGAGCGTCTCGCCGCGGCGCGGCAGCAGGCGGATTGGATCACCGAT
GGCGATCCGTCGAAGGGCTCGCCAGCGGCAAGCGTCATGATCTCGAAAATCTGCAGACG
CGCATCAAGGCGCTCTCGGGCAAGGTCGTACCATCAAGGCCGGCGGACTCTCGGATATT
CTGATCAAGGAACGCATGCAACGCATCGAGAATGCCCTCGCCTCGGCGCGCGCCGCGCGC
AGCGACGGCGTCGTGCGCGGCGGCGGCGTCGGGCTCTATCGCGCGCGCGCCGCTCTCACC
GAAGCGACGGGCGACACTCTCGATCAGACCTATGGAATCGCTATTGTGCGCGCGGCGCTC
GACGAGCCGATCCGTCGCATCGCCGCCAATGCGGGGCGCGATGCGCATGAGTTTTTATTC
GAGCTCAAACGTTTGAACGATGATTTCTGGGGCATGGACATGCGCAGCGGCGAATGCGGC

GATCTCTATGCGGCGGGCGTCATCGATCCGGCGCGGTACGCGTCTCGCATTGCGCAAC
GCCGTCGCCACCGCATCGTCATTGATGACCGTCGAATGCGCGGTCACTCATATTCCGCCG
AGCGATCCGACATACGGCTTCGATCCGCATCTCGCCGCTGCGACGCGTGAAGATCCGCGC
AGCTGA